

## Presenilin-1 and -2 Are Molecular Targets for $\gamma$ -Secretase Inhibitors\*

Received for publication, June 21, 2000

Published, JBC Papers in Press, July 27, 2000, DOI 10.1074/jbc.M005430200

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Presenilins are integral membrane protein involved in the production of amyloid  $\beta$ -protein. Mutations of the presenilin-1 and -2 gene are associated with familial Alzheimer's disease and are thought to alter  $\gamma$ -secretase cleavage of the  $\beta$ -amyloid precursor protein, leading to increased production of longer and more amyloidogenic forms of A $\beta$ , the 4-kDa  $\beta$ -peptide. Here, we show that radiolabeled  $\gamma$ -secretase inhibitors bind to mammalian cell membranes, and a benzophenone analog specifically photocross-links three major membrane polypeptides. A positive correlation is observed among these compounds for inhibition of cellular A $\beta$  formation, inhibition of membrane binding and cross-linking. Immunological techniques establish N- and C-terminal fragments of presenilin-1 as specifically cross-linked polypeptides. Furthermore, binding of  $\gamma$ -secretase inhibitors to embryonic membranes derived from presenilin-1 knockout embryos is reduced in a gene dose-dependent manner. In addition, C-terminal fragments of presenilin-2 are specifically cross-linked. Taken together, these results indicate that potent and selective  $\gamma$ -secretase inhibitors block A $\beta$  formation by binding to presenilin-1 and -2.

$\beta$ -Amyloid precursor protein ( $\beta$ APP)<sup>1</sup> is a transmembrane protein that undergoes processing to A $\beta$  by proteolytic activities known as  $\beta$ - and  $\gamma$ -secretases (for review, see Refs. 1–3). The  $\beta$ -secretase cleavage occurs in the extracellular domain by a recently identified aspartyl protease variously termed BACE, memapsin, and Asp2 (4–9), whereas the heterogeneous  $\gamma$ -secretase cleavage occurs in the transmembrane domain (2, 10). Dominant mutations in either of the two human presenilin (PS-1 and PS-2) genes lead to familial Alzheimer's disease (AD). PS-1 and -2 are polytopic membrane proteins (for review, see Refs. 11–13). Presenilins are proteolytically processed. *In vivo*, only small amounts of the holoprotein can be detected, primar-

ily in the nuclear envelope, whereas 30-kDa N-terminal and 20-kDa C-terminal fragments of presenilin are observed in all mammalian tissues and cell lines analyzed so far. Coimmunoprecipitation experiments revealed that presenilin fragments are assembled into a high molecular weight complex together with other proteins (for review see 11–13). The proposed mechanism through which the presenilin mutations cause AD is an alteration in the predominant  $\gamma$ -secretase cleavage site which increases the amount of the longer, more amyloidogenic A $\beta$  1–42(43) fragments produced (11–13). A null mutation of the mouse PS-1 selectively reduces  $\gamma$ -secretase activity (14), and site-directed mutagenesis of PS-1 and PS-2 at two conserved aspartyl residues, which resemble the catalytic center of aspartyl proteases, also reduces  $\gamma$ -secretase activity (15, 16). These observations indicate that PS-1 and PS-2 either stimulate the activity of  $\gamma$ -secretase by trafficking to appropriate cellular compartments, serve as cofactors of the  $\gamma$ -secretase, or are  $\gamma$ -secretase themselves.

Here, we report that a series of potent and selective  $\gamma$ -secretase inhibitors bind to mammalian cell membranes and specifically cross-link to three major polypeptides. Immunological techniques identified PS-1 and PS-2 as the major, specifically cross-linked polypeptides. These results indicate that this series of potent and selective  $\gamma$ -secretase inhibitors blocks A $\beta$  formation by binding to PS-1 and PS-2

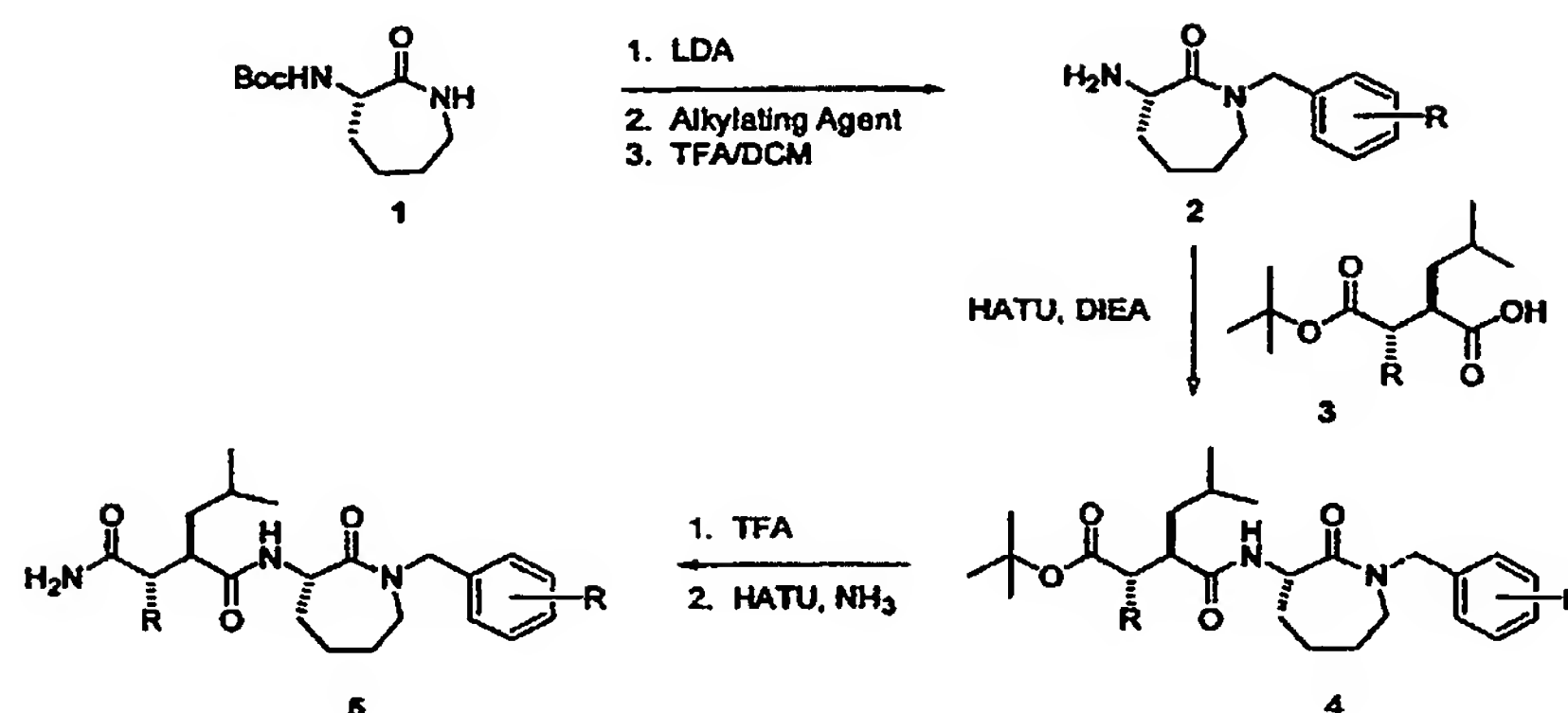
### EXPERIMENTAL PROCEDURES

**Binding Assays**—Binding assays using [<sup>3</sup>H]Compound A (see Table I; 87.5 Ci/mmol) were performed using standard methods (17). THP-1 cells were grown in Spinner cultures in RPMI 1640 containing L-glutamine (Life Technologies, Inc.) and 10  $\mu$ M  $\beta$ -mercaptoethanol to a density of  $5 \times 10^6$ /ml. Cells were harvested by centrifugation, and cell pellets were quick frozen in dry ice/ethanol and stored at  $-70^\circ\text{C}$  prior to use. The pellets of approximately  $2 \times 10^8$  THP-1 cells were homogenized in 10 ml of either 50 mM Hepes, pH 7.0, or 50 mM Tris, pH 7.4, at  $4^\circ\text{C}$ , using a Brinkmann Polytron at setting 6 for 10 s. The homogenate was centrifuged at  $48,000 \times g$  for 12 min, and the resulting pellet was washed by repeating the homogenization and centrifugation steps. The final cell pellet was resuspended in buffer to yield a protein concentration of approximately 0.5 mg/ml. Assays were initiated by the addition of 150  $\mu$ l of membrane suspension to 150  $\mu$ l of assay buffer containing 0.064  $\mu$ Ci of radioligand and various concentrations of unlabeled compounds. Binding assays were performed in duplicate in polypropylene 96-well plates (Costar, Cambridge, MA) in a final volume of 0.3 ml of 50 mM Hepes, pH 7.0, or 50 mM Tris, pH 7.4, containing 5% (v/v) dimethyl sulfoxide. Nonspecific binding was defined in the presence of 300 nM Compound E or as indicated in the figure legends. After incubating at  $23^\circ\text{C}$  for 1.3 h (or as indicated in the figure legends), the separation of bound from free radioligand was accomplished by filtration over GFF glass fiber filters (Inotech Biosystems International, Lansing, MI) presoaked in 0.3% ethyleneimine polymer solution using an Inotech cell

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<sup>1</sup> The abbreviations used are:  $\beta$ APP,  $\beta$ -amyloid precursor protein; A $\beta$ , 4-kDa amyloid  $\beta$ -peptide; PS, presenilin; AD, Alzheimer's disease; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HPLC, high performance liquid chromatography.



SCHEME 1. Synthesis of  $\gamma$ -secretase inhibitors.

harvester. Filters were washed three times with 0.3 ml of ice-cold phosphate-buffered saline, pH 7.0, containing 0.01% Triton X-100. Filters were assessed for radioactivity by liquid scintillation counting using a Packard 2500 TR (Packard Instrument Co., Downers Grove, IL).  $K_i$  values of competing compounds were derived through Cheng-Prussoff correction of  $IC_{50}$  values calculated using the program GraphPad Prism by GraphPad Software (San Diego, CA).

**Cross-linking Studies**—THP-1 membranes for cross-linking studies were prepared as outlined for the binding studies. Membranes were preincubated for 30 min at room temperature with labeled Compound C in the presence or absence of unlabeled competitors. Photolysis at 365 nm was performed for 1 h at room temperature. The membranes were harvested by centrifugation ( $40,000 \times g$  for 20 min), resuspended in SDS sample buffer, and analyzed by SDS-PAGE (18). The following experiments were performed to optimize the cross-linking conditions. (i) The membrane protein concentration was varied from 1 to 10 mg/ml. (ii) Ligand concentrations were varied from 10 to 120 nM. (iii) Cross-linking temperature was varied between 4 °C and room temperature. Overall, the specifically labeled polypeptides patterns were not altered by any of the above procedures. In addition, the intensity of the labeling increased between 5 and 30 min of UV illumination and plateaued thereafter. The cross-linking experiments presented in this manuscript were all performed with 1 h of illumination. Similar gel patterns were obtained when samples were analyzed under nonreducing and reducing conditions. However, boiling for extended periods of time resulted in multimerization and disappearance of specifically labeled polypeptides.

**Immunoprecipitation Studies**—THP-1 membranes (10 mg/ml) were incubated with radiolabeled Compound C (120 nM) for 30 min at room temperature followed by photolysis at room temperature for 1 h. The membranes were harvested by centrifugation ( $40,000 \times g$  for 20 min) and extracted (10 mg of membrane proteins/ml of extraction buffer) with 2% CHAPS, 50 mM Tris buffer, pH 7.4, for 1 h at 4 °C in the presence of complete protease inhibitors (one tablet of protease inhibitors/25 ml of extraction buffer; Roche Molecular Biochemicals). The membrane extract was centrifuged at  $40,000 \times g$  for 20 min. Similar results were obtained with membrane extracts centrifuged at  $100,000 \times g$  for 1 h. Comparison of different extraction procedures revealed that 2% CHAPS quantitatively solubilized the cross-linked polypeptides compared with SDS sample buffer, which was assumed to solubilize the membrane proteins completely. The membrane extract was diluted with an equal volume of distilled water and preabsorbed (1 h; 4 °C) with normal mouse serum (10  $\mu$ l/ml of extract) and goat anti-mouse IgG-Sepharose (Sigma). The beads were removed by centrifugation followed by the addition of antibodies to PS-1 (10  $\mu$ g/ml) and goat anti-mouse IgG-Sepharose for 16 h at 4 °C. The beads were washed three times with 1% CHAPS in binding buffer (1 ml each wash) and three times with phosphate-buffered saline (1 ml each wash). Bound proteins were eluted by boiling (approximately 3 min) in SDS sample buffer and analyzed by SDS-PAGE (18) and fluorography. For immunoprecipitation of PS-2 fragments, the CHAPS extract was preabsorbed with normal rabbit serum, and protein A-Sepharose (Sigma) was used instead of the anti-mouse IgG-Sepharose.

**Large Scale Affinity Purification of Cross-linked Polypeptides**—Normal mouse IgG (Sigma) or anti-PS-1 IgG was immobilized to an agarose matrix (AminoLink Plus Immobilization Kit) at 2 mg/ml of beads according to the manufacturer's instructions (Pierce). THP-1 membrane extracts (5 mg/ml membrane proteins) in 1% CHAPS, 25 mM Tris, pH 7.4, were applied by batch absorption (16 h; room temperature) to the normal mouse IgG column. The nonbound fraction was recovered by

centrifugation and applied by batch absorption (16 h; room temperature) to the PS-1 column. Both columns were washed extensively in parallel with 1% CHAPS, 25 mM Tris, pH 7.4, followed by 1% CHAPS in distilled water and eluted (15 min; room temperature) with 0.1 M glycine, 0.5% CHAPS, pH 2.3. The elution fractions were neutralized immediately by the addition of 1 M Tris, pH 9.5. For immunoblotting analysis, polypeptides were transferred to nitrocellulose membranes and analyzed as described (19). Prestained molecular size markers were obtained from Life Technologies, Inc.

**Determination of A $\beta$  Levels by Enzyme-linked Immunosorbent Assay**—The ability of compounds to lower A $\beta$  secretion was tested using Chinese hamster ovary cells overexpressing wild-type  $\beta$  APP as described previously (20) and Roach *et al.*<sup>2</sup> Briefly, confluent cells were incubated with a range of concentrations of compounds for 16 h in serum-free medium containing 0.2% bovine serum albumin. A $\beta$  1–40 in the resultant conditioned media was quantified by sandwich enzyme-linked immunosorbent assay using a position 40 neopeptide-specific monoclonal antibody and a biotinylated monoclonal antibody directed to A $\beta$  residues 10–20.

**Compound Synthesis and Labeling**—Compounds A–D and F and G were prepared according to the general synthesis strategy shown in Scheme 1 following the methods described in PCT application WO 00/07995. *t*-Butoxycarbonyl-caprolactam 1 was deprotected with lithium diisopropylamide and alkylated with a benzylic halide to introduce the diaryl ether or benzophenone substituent. In the case of Compound G, 3-iodobenzyl bromide was employed, which allowed the subsequent construction of the biaryl group using Suzuki coupling. The *t*-butoxycarbonyl group was removed using trifluoroacetic acid and the resulting amine was coupled to a substituted succinic acid derivative using the coupling agent hexafluorophosphate in the presence of diisopropylethylamine. The succinic acid derivatives were prepared using Evans' oxazolidinone methodology according to published procedures (21 and WO 97/18207). After coupling, the *t*-butyl ester was removed using trifluoroacetic acid and the primary amide was prepared using hexafluorophosphate and excess gaseous ammonia. Compound D was prepared in an analogous fashion using 3-(*S*)-amino-1-methyl-5-phenyl-1,3-dihydro-benzo[*e*](1,4)diazepin-2-one as described in PCT application WO 00/07995. Compound E was prepared according to WO 98/28268.

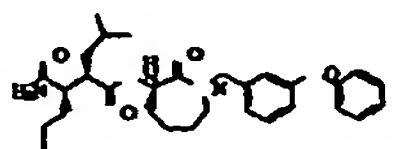
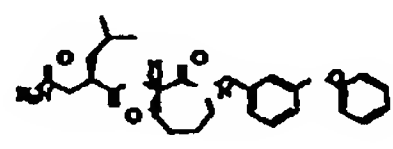
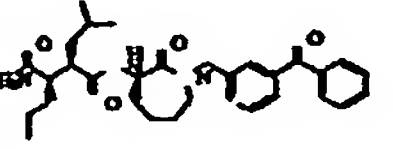
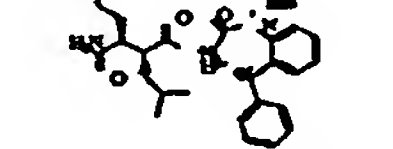
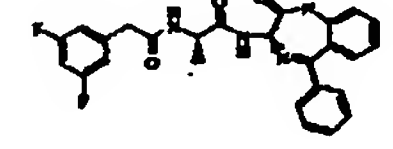
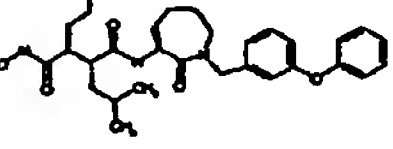
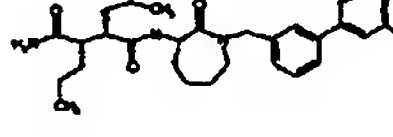
[<sup>3</sup>H]Compound C was labeled at the propyl site chain by reduction in the presence of tritium gas. Briefly, to a reaction vessel containing a stirred solution of (2-allyl-N<sup>4</sup>-[1-(3-benzoyl-benzyl)-2-oxo-azepan-3-yl]-3-isobutyl-succinamide, 10 mg) in tetrahydrofuran (2 ml) was added 10% palladium on carbon (10 mg). The reaction vessel was evacuated and degassed via a freeze-thaw evacuation cycle and then exposed to tritium gas (50 Ci). After 30 min, unreacted tritium gas was removed, and the catalyst was filtered from the reaction mixture. The solvent was removed *in vacuo*, and the labile tritium was further removed by evaporation with methanol (three times 2 ml). The residue was dissolved in 0.1% aqueous trifluoroacetic acid/methanol (50:50; 10 ml) and purified on a reversed-phase HPLC column (Zorbax SB-C18, 25  $\times$  0.92 cm) using 50% acetonitrile in 0.1% aqueous trifluoroacetic acid as the mobile phase (flow rate 2 ml/min). Fractions containing the product were pooled, and the solvent was removed by rotary evaporation. The product was dissolved in ethanol (145 mCi, 15.5 mCi/ml). HPLC analysis (Vydac protein-peptide C18, 0.46  $\times$  25 cm, 30% methanol in 0.05% aqueous

<sup>2</sup> A. H. Roach, D. Seiffert, L. A. Thompson, B. Cordell, J. N. Higaki, R. E. Olson, and R. Zaczek, unpublished observations.



TABLE I  
Molecules (Compounds A-G) in formation of A $\beta$ -Peptides

Binding assays using [ $^3$ H]Compound A (87.5 Ci/mmol) were performed using THP-1 membranes (see "Experimental Procedures").  $K_i$  values of competing compounds were derived through Cheng-Prusoff correction of IC<sub>50</sub> values calculated using the program GraphPad Prism. The ability of compounds to lower A $\beta$  secretion was tested using Chinese hamster ovary cells overexpressing wild-type  $\beta$ APP. Briefly, confluent cells were incubated with a range of concentrations of compounds for 16 h in serum-free medium containing 0.2% bovine serum albumin. A $\beta$  X-40 in the resultant conditioned media was quantified by sandwich enzyme-linked immunosorbent assays using a position 40 neopeptide-specific mAB and a biotinylated mAB directed to A $\beta$  10-20.

	Structure	A beta Production IC <sub>50</sub> (nM)	[ $^3$ H]RE987 Binding K <sub>i</sub> (nM)
Compound A		40	16
Compound B		52000	7900
Compound C		90	51
Compound D		13	2.6
Compound E		0.3	0.73
Compound F		265	92
Compound G		22	5.8

trifluoroacetic acid for 5 min, 15-min gradient to 95% methanol in 0.05% aqueous trifluoroacetic acid, 1 ml/min) indicated the radiochemical purity to be greater than 99%. MS (FAB):  $m/z$ , 526 (most abundant ion),  $^3$ H NMR (proton decoupled, 320 MHz, CDCl<sub>3</sub>): multiplets at  $\delta$  0.83, 1.21.

**Miscellaneous**—The N-terminal monoclonal antibody to PS-1 (clone 614) was raised to a glutathione S-transferase fusion protein expressed in bacteria containing the N-terminal 77 residues of PS-1. The C-terminal monoclonal antibody (clone 361) was obtained by immunizing mice with a synthetic peptide to PS-1 residues 309-331. Rat anti-PS-1 N-terminal monoclonal antibody and PS-1 polyclonal antibodies were obtained from Chemicon. Antibodies to PS-2 were kindly provided by Bart De Strooper. Embryonic tissue from PS-1 and PS-2 gene target animals was provided by Bart De Strooper and Paul Saftig.

## RESULTS

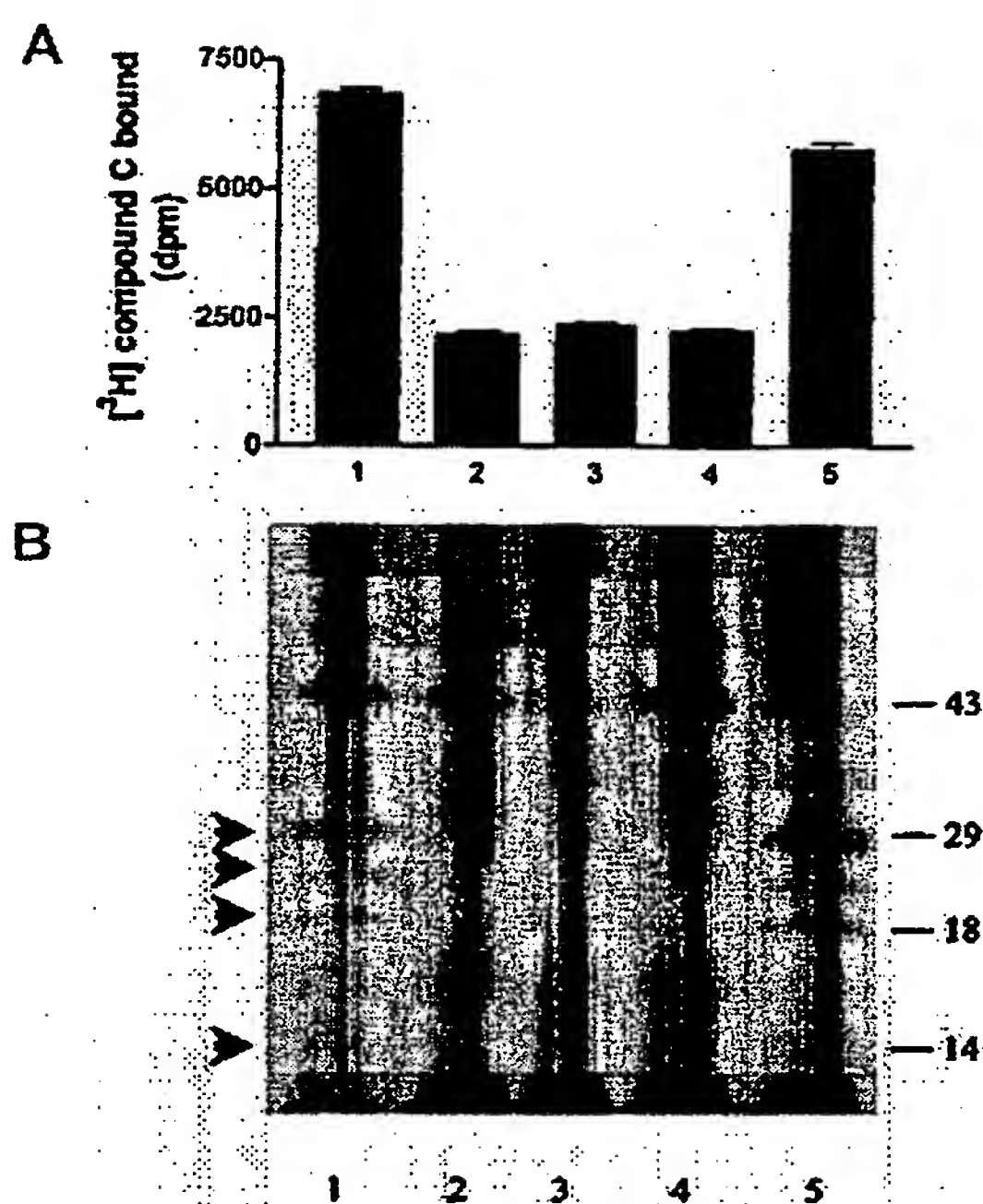
**Correlation between Cellular Potency and Membrane Binding of  $\gamma$ -Secretase Inhibitors**—We have identified a series of small molecules (Compounds A-D, F, and G) which inhibit the formation of the A $\beta$  1-40 and 1-42 peptides in  $\beta$ APP-transfected and nontransfected mammalian cells with nanomolar potencies<sup>2</sup> (Table I). For comparison, we have also characterized Compound E, described in the patent literature (PCT application WO 98/28268). A $\beta$  formation was specifically inhibited without any signs of cellular toxicity in *in vitro* assays.<sup>2</sup> These compounds have been classified as inhibitors of  $\gamma$ -secretase by virtue of their ability to induce the increased accumulation of the C-terminal 99-amino acid fragment of  $\beta$ APP

(CT99),<sup>2</sup> the substrate for  $\gamma$ -secretase action which results from  $\beta$ -secretase processing (1, 3, 11-13).

[ $^3$ H]Compound A bound specifically and reversibly to rat brain membranes (not shown). A survey of established mammalian cell lines was performed to identify a high expressing, easily grown cellular source for membrane binding and molecular target identification. Of the eight lines analyzed, membranes from a human macrophage cell line, THP-1, possessed the highest specific binding capacity ( $B_{max}$ ). The  $B_{max}$  was  $1,057 \pm 138$  ( $n = 2$ ) fmol/mg THP-1 membrane protein. Association kinetics using THP-1 membranes revealed a  $K_d$  of 2.4 nM and a  $t_{1/2}$  of association of  $0.75 \pm 0.49$  min ( $n = 2$ ). In addition, Compound A blocked A $\beta$  formation in THP-1 cells (data not shown). These results suggest that THP-1 cells contain the pharmacological site for A $\beta$  lowering and membrane binding.

The correlation between cellular potency (IC<sub>50</sub> for inhibition of A $\beta$  formation in Chinese hamster ovary cells overexpressing  $\beta$ APP) and  $K_i$  for membrane binding is indicated in Table I for seven compounds ranging over 4 log units in affinity; we observed a significant correlation ( $r^2 = 0.96$ ;  $p < 0.0001$  (two-tailed  $t$  test)) for displacement of a radiolabeled  $\gamma$ -secretase inhibitor and cellular potency for A $\beta$  lowering. A similar correlation was observed using Compound C (a photochemical cross-linker) or D as ligand. Moreover, a positive correlation between cellular potency and membrane binding was also observed in human cell lines expressing endogenous  $\beta$ APP, IMR32 neuroblastoma cells, and in human embryonic kidney (HEK 293) cells overexpressing  $\beta$ APP (data not shown). These results strongly suggest that the membrane preparations contain the intact molecular target of  $\gamma$ -secretase inhibitors.

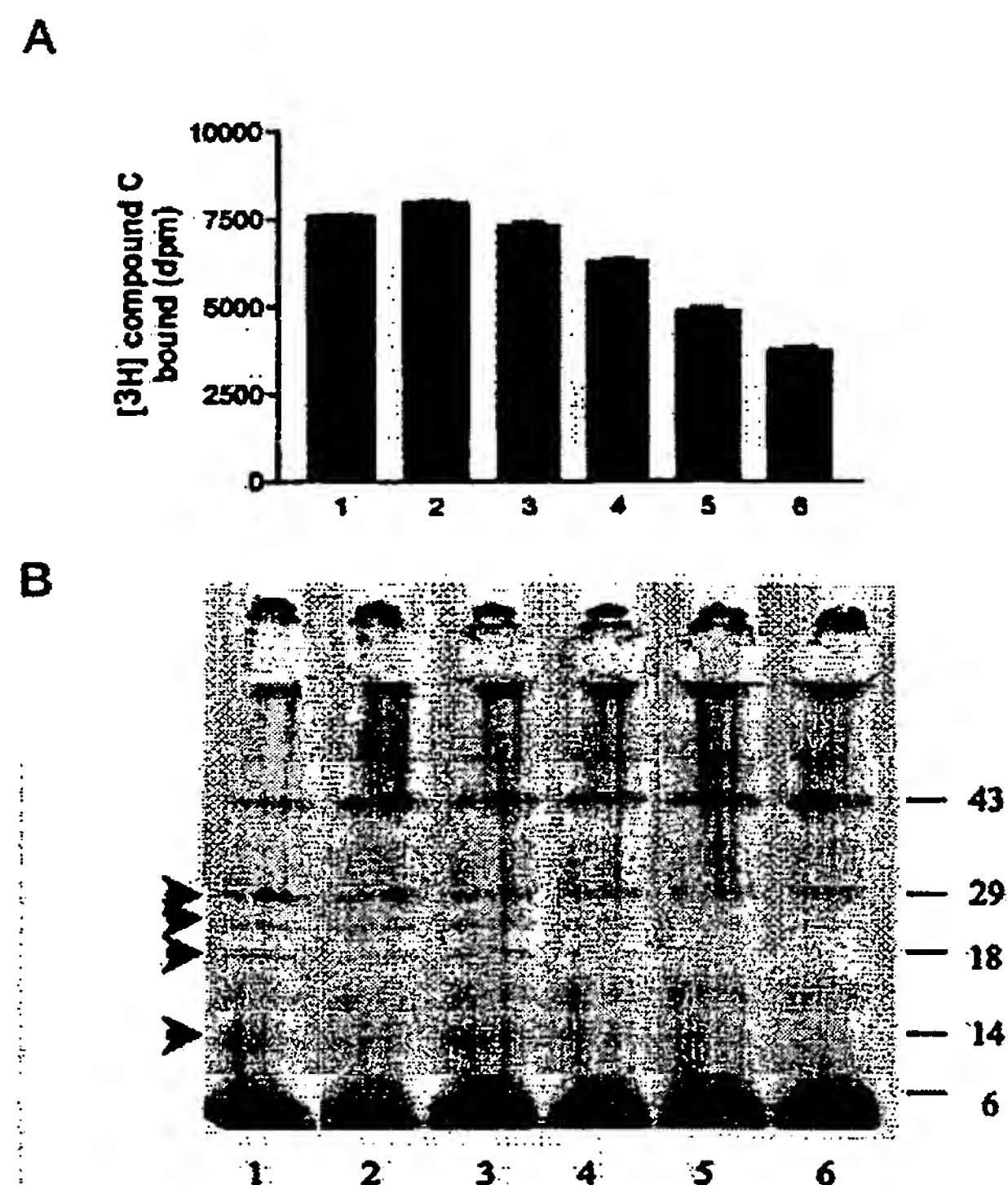
**Correlation between Membrane Binding and Photoaffinity Cross-linking**—Photocross-linking experiments were performed to identify the target of these  $\gamma$ -secretase inhibitors. THP-1 membranes were preincubated with radiolabeled Compound C in the absence or presence of the indicated unlabeled competitors. Membranes were analyzed by binding (Fig. 1A) or by cross-linking after exposure to UV light (Fig. 1B). After cross-linking, membranes were harvested by centrifugation, resuspended in SDS sample buffer, fractionated by SDS-PAGE, and analyzed by fluorography (Fig. 2B). Several major polypeptides of 43, 30, 25, 20, and 14 kDa (as well as several minor species) were identified by the cross-linking approach. The reduction in specific binding in the binding assay by competing with unlabeled inhibitors (panel A) correlated with the reduction in cross-linking intensity of the 30-, 25-, 20-, and 14-kDa polypeptides (panel B). No additional specifically labeled polypeptides were observed when cross-linked polypeptides were analyzed by 15% polyacrylamide gels (not shown). Importantly, a chemically similar, yet much less potent compound (Compound B) failed to compete for cross-linking of specifically labeled bands of 30, 25, 20, and 14 kDa (Fig. 1B, lane 5). Also, Compound B did compete for major cross-linking band at 43 kDa, whereas the active Compounds A and E did not compete for the 43-kDa band. Taken together, these observations allow us to classify the 43-kDa band as nonspecifically labeled. Moreover, the specific cross-linking to the 30-, 25-, and 20-kDa bands was also observed in HEK 293 cells (data not shown). Additional experiments established a dose-dependent reduction in specific binding and cross-linking intensity. For example, for each of the seven compounds analyzed, the corresponding pair of dose-response curves for inhibition of binding and reduction in photocross-linking was indistinguishable for compounds ranging over 4 log potency (0.3-7,900 nM). For example, the parallel reduction of binding and cross-linking intensity of the 30-, 25-, and 20-kDa bands is indicated for Compound D in



**FIG. 1. Correlation between binding and photoaffinity cross-linking in THP-1 membranes.** THP-1 membranes were incubated with 10 nM [ $^3$ H]Compound C (83.4 Ci/mmol) and analyzed by filtration binding assay (panel A) or, after cross-linking, by SDS-PAGE and fluorography (panel B). Competition experiments with unlabeled compounds (1  $\mu$ M) established that only four of the polypeptides, indicated by the arrowheads (30, 25, 20, and 14 kDa) were specifically labeled. Note that reduction in cross-linking (panel B) correlates positively with reduction in binding (panel A). Lane 1, dimethyl sulfoxide control; lane 2, Compound A; lane 3, Compound C; lane 4, racemic mixture of Compound E; lane 5, Compound B.

**Fig. 2.**

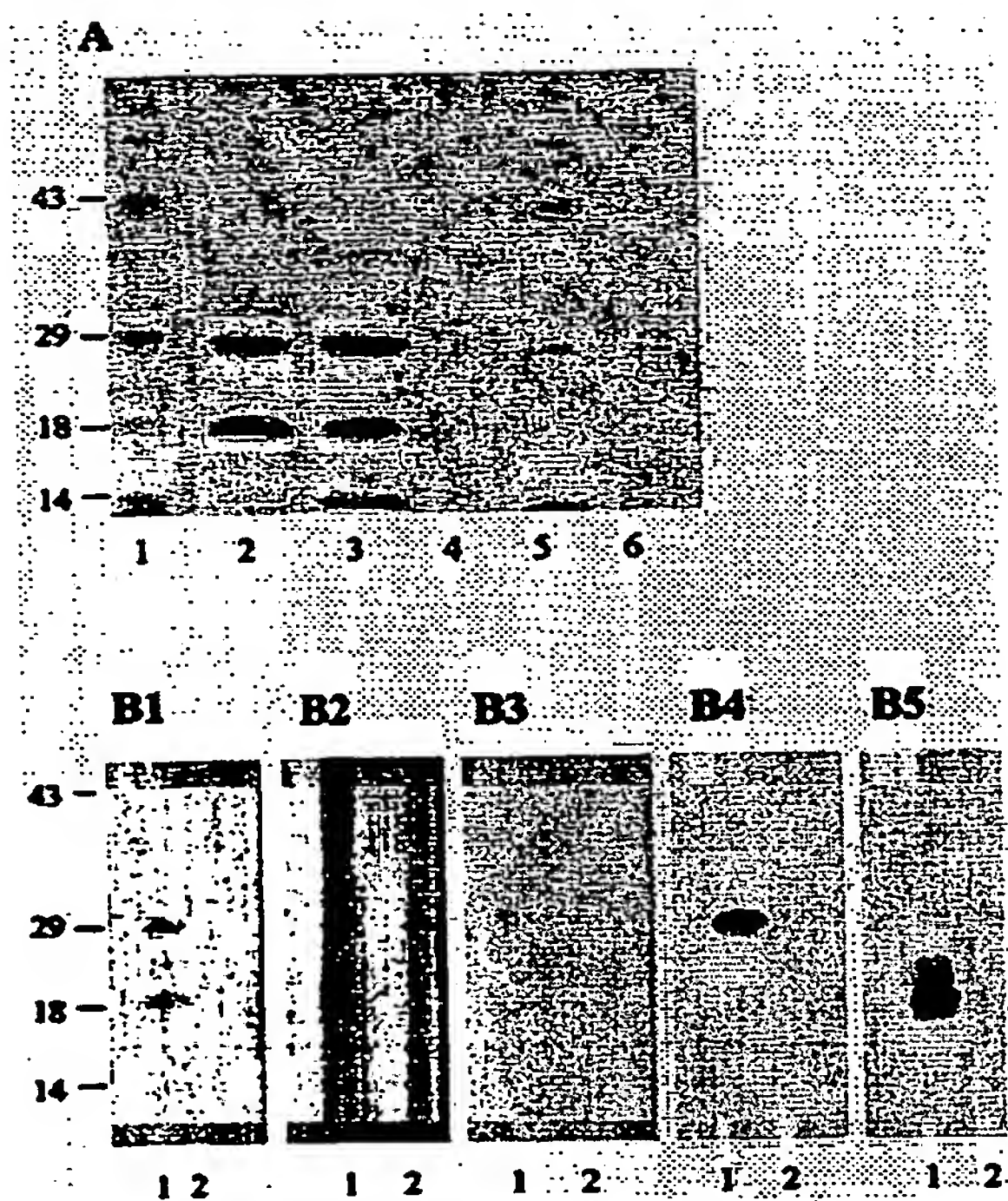
**Identification of the Molecular Target of  $\gamma$ -Secretase Inhibitors**—The molecular sizes of the cross-linked polypeptides (11–13) are, in part, consistent with the hypothesis that presenilins are the molecular target. To determine whether Compound C cross-linked to presenilins, membranes were incubated with tritiated Compound C, cross-linked, extracted with 2% CHAPS, and analyzed by immunoprecipitation using antibodies to PS-1. Extraction of membranes with 2% CHAPS quantitatively solubilized the specifically cross-linked polypeptides. Regardless of subunit specificity, antibodies to PS-1 are expected to immunoprecipitate both N- and C-terminal PS-1 fragments because CHAPS extraction does not dissociate the PS-1 complex (22, 23). The major labeled polypeptides of 30 and 20 kDa coimmunoprecipitated with antibodies to PS-1 (Fig. 3). Similar immunoprecipitation patterns were observed with three commercially available antibodies to PS-1 (data not shown; see antibody listing under “Experimental Procedures”). The immunoprecipitation was specific, as shown by the disappearance of radiolabeled bands when cross-linking was performed in the presence of excess Compound E or when the PS-1 antibody was replaced by normal mouse IgG. In addition, the PS-1 antibodies immunoprecipitated only the specifically cross-linked polypeptides, whereas nonspecifically labeled bands were not recovered in the immunoprecipitate. The latter finding rules out precipitation of incompletely solubilized membrane fragments. It should be noted that the antibody to the C-terminal loop region of PS-1 also immunoprecipitated the 14-kDa polypeptide (Fig. 3A, lane 3). Similar immunoprecipitation patterns were observed using HEK 293 cell membranes (data not shown).



**FIG. 2. Correlation between binding and photoaffinity cross-linking in THP-1 membranes for Compound D.** THP-1 membranes were incubated with 10 nM [ $^3$ H]Compound C (83.4 Ci/mmol) in the presence of the indicated concentrations of Compound D and analyzed by filtration binding assay (panel A) or, after cross-linking, by SDS-PAGE and fluorography (panel B) (see Fig. 1). Note that the reduction in cross-linking (panel B) to bands of 30, 25, and 20 kDa (arrowheads) correlates positively with reduction in binding (panel A). Lane 1, dimethyl sulfoxide control; lane 2, 0.3 nM Compound D; lane 3, 1 nM Compound D; lane 4, 3 nM Compound D; lane 5, 10 nM Compound D; lane 6, 30 nM Compound D. The mobility of molecular weight markers is indicated.

When membranes were extracted under conditions known to disrupt the PS-1 complex (in the presence of 1% SDS), the N-terminal PS-1 antibody immunoprecipitated only the 30-kDa band; likewise, the C-terminal antibody precipitated only the 20-kDa band (not shown). Large scale affinity purification was performed using the C-terminal PS-1 antibody or IgG derived from normal mouse serum bound to Sepharose (Fig. 3B). The specifically cross-linked bands of 30, 25, and 20 kDa bound to and eluted from the anti PS-1 IgG column, but not normal mouse IgG column. The elution fractions of both columns were analyzed by fluorography (Fig. 3, B1), and by silver staining (Fig. 3, B2) followed by fluorography of the dried silver-stained gel (Fig. 3, B3). The 30- and 20-kDa silver-stained polypeptides were also positive in the fluorography. Moreover, immunoblotting analysis established that the 30-kDa band reacted with antibodies to the N terminus of PS-1 (Fig. 3, B4), whereas the 20-kDa band reacted with antibodies to the C terminus of PS-1 (Fig. 3, B5). It should be noted that traces of the 25-kDa polypeptide copurified with the PS-1 C-terminal antibody (Fig. 3, B1); however, this band was not detected by immunoblotting using N- or C-terminal PS-1 antibodies. These observations suggest that the 25-kDa band copurified with the PS-1 complex. The involvement of PS-1 in compound binding was confirmed in binding studies using membranes from PS-1 knockout embryos (14). A gene dose-dependent reduction in binding was observed (Fig. 4). It should be noted that even in the

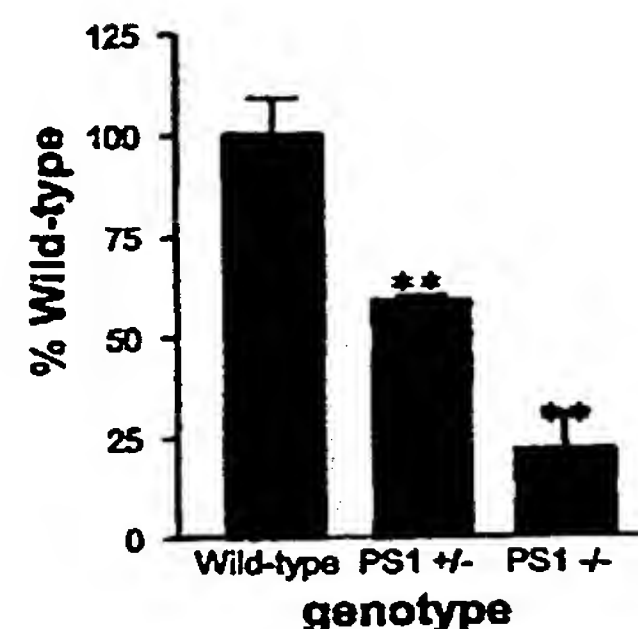




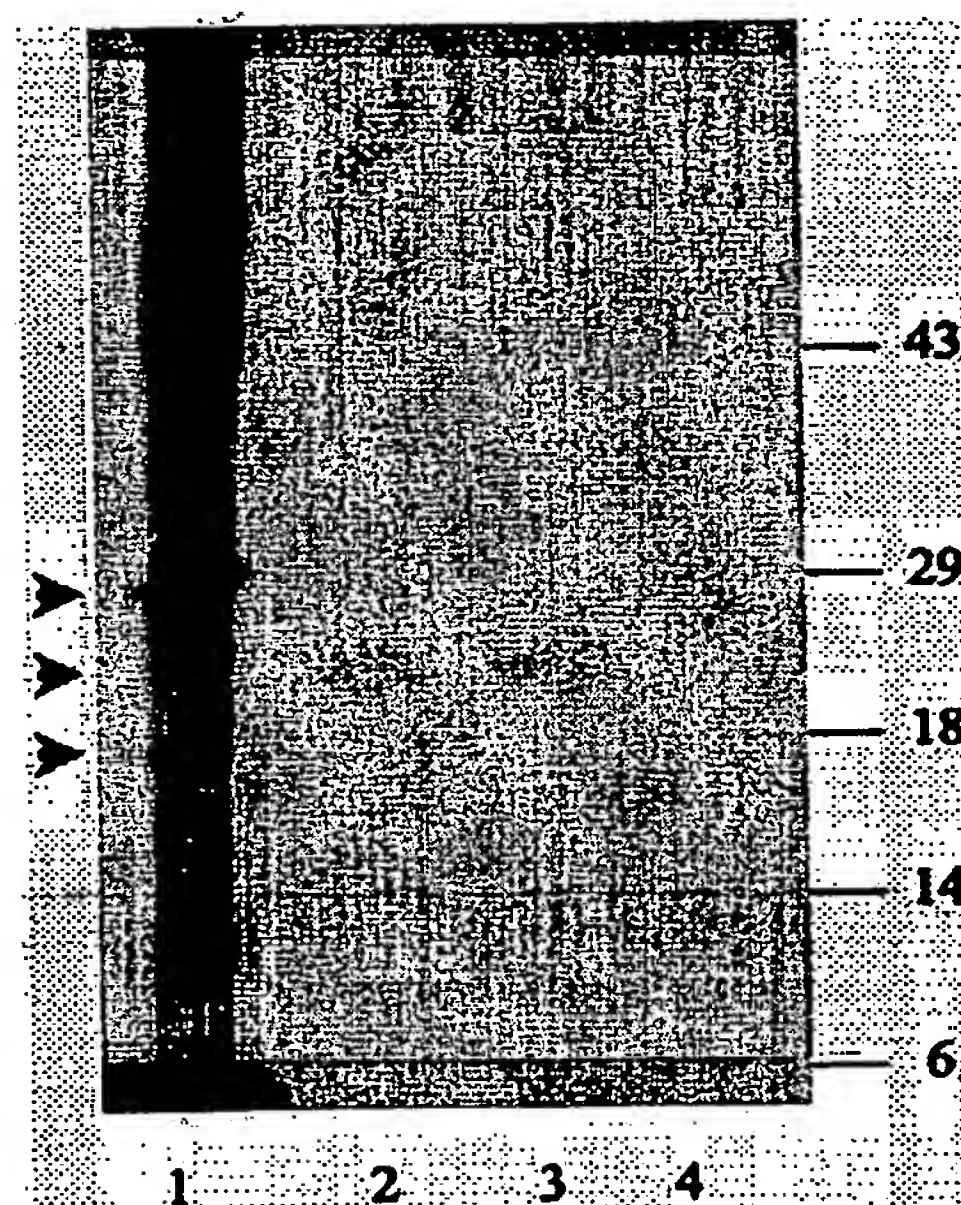
**FIG. 3. Immunopurification identifies PS-1 as the molecular target for  $\gamma$ -secretase inhibitors.** *Panel A*, immunoprecipitation of radiolabeled polypeptides with PS-1 antibodies. THP-1 membranes were incubated with 120 nM [ $^3$ H]Compound C in the absence or presence of Compound E. After cross-linking, membranes were extracted with CHAPS and analyzed by immunoprecipitation using anti-human PS-1 IgG (to either the N terminus or C-terminal loop). Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE (11%) and fluorography. The band below 14 kDa in lanes 1 and 5 represents free cross-linker comigrating with the dye front. *Lane 1*, CHAPS extract, no competition; *lane 2*, immunoprecipitation with PS-1 N-terminal monoclonal antibody; *lane 3*, immunoprecipitation with PS-1 C-terminal loop antibody; *lane 4*, immunoprecipitation with normal mouse IgG control; *lane 5*, CHAPS extract, specific cross-linking competed by unlabeled Compound E (racemic mixture); *lane 6*, immunoprecipitation PS-1 N-terminal monoclonal antibody using CHAPS extract from cross-linking competed by unlabeled Compound E (racemic mixture). *Panel B*, affinity purification of photoaffinity cross-linked PS-1. THP-1 membranes were photoaffinity cross-linked as in *panel A*. The resulting CHAPS extracts were applied to a normal mouse IgG column followed by a PS-1 (C-terminal loop) antibody column. The columns were washed extensively and eluted with 0.5% CHAPS, 0.1 M glycine, pH 2.3. The elution fractions were analyzed by fluorography (*B1*) and silver staining (*B2*). Note the presence of the 30- and 20-kDa band in the elution of the PS-1 (*lane 1*) but not the mouse IgG column (*lane 2*). Fluorography of the silver-stained gel revealed that the 30- and 20-kDa bands contain the majority of radioactivity (*B3*). The 30-kDa band was detected by immunoblotting with monoclonal antibodies to the N terminus of PS-1 (*B4*), whereas the 200-kDa band was detected by monoclonal antibodies to the C-terminal loop of PS-1 (*B5*). In *panels B1-B5*, *lane 1* is the elution from anti-PS-1-Sepharose, and *lane 2* is the elution from normal mouse IgG-Sepharose.

absence of PS-1 expression, residual specific binding of approximately 25% of the wild-type animals was observed. These results suggest that PS-1 is not the only binding site for the compounds under study. Incorporation of radioactivity into the 14-kDa band varied among several THP-1 and HEK 293 cell preparations. Based on the observed variability, this band was not a focus of this study.

The involvement of PS-2 in binding and cross-linking was further analyzed (Fig. 5). Membranes from THP-1 cells were cross-linked and extracted as for the PS-1 experiments and analyzed by immunoprecipitation using antibodies to PS-2. The extract contained the major specifically labeled polypeptides of



**FIG. 4. Binding of labeled  $\gamma$ -secretase inhibitors to PS-1 knockout embryonic membranes.** Embryos from PS-1 knockout mice were tested at E17-18. Whole embryos were homogenized in buffer (10% w/v), and membranes were prepared. The final pellet was resuspended in buffer at a protein concentration of approximately 8-10 mg/ml original wet weight. Radioligand binding assays were performed as described under "Experimental Procedures," the ligand used was [ $^3$ H]Compound D (86 Ci/mmol). Specific binding was determined using 0.064  $\mu$ Ci of [ $^3$ H]Compound D and 300 nM Compound E to define nonspecific binding. Data are mean values of four animals for each group and are expressed as a percentage of specific binding observed in wild type embryos; \*\* indicates  $p < 0.01$  as determined by one-way analysis of variance and Dunnett's tests.



**FIG. 5. Immunoprecipitation identifies PS-2 as the molecular target for  $\gamma$ -secretase inhibitors.** THP-1 membranes were incubated with 120 nM [ $^3$ H]Compound C. After cross-linking, membranes were extracted with CHAPS and analyzed by immunoprecipitation using anti-human PS-2 IgG (to either the N terminus or C-terminal loop). Bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE (11%) and fluorography. *Lane 1*, CHAPS extract; *lane 2*, immunoprecipitation with PS-2 N-terminal antibody; *lane 3*, immunoprecipitation with PS-2 C-terminal loop antibody; *lane 4*, immunoprecipitation with normal rabbit IgG control.

30, 25, and 20 kDa (Fig. 5, *lane 1*). Immunoprecipitation with both N- and C-terminal specific polyclonal antibodies to PS-2 identified the 25-kDa fragment as PS-2 related (Fig. 5, *lanes 2* and 3). Control immunoprecipitation experiments using normal rabbit IgG established that the immunoprecipitation was specific (Fig. 5, *lane 4*). The 25-kDa fragment was the only band specifically stained in THP-1 membrane extracts by immunoblotting using the PS-2 C-terminal antibody (not shown). More-



over, the 25-kDa band comigrated with the PS-2 C-terminal fragment derived by *in vitro* transcription and translation of a 5'-truncated PS-2 cDNA (not shown). Taken together, these results indicate that potent and selective  $\gamma$ -secretase inhibitors block A $\beta$  formation by binding to PS-1 and -2.

#### DISCUSSION

The studies presented here provide evidence that the described small molecule  $\gamma$ -secretase inhibitors bind to PS-1 and PS-2. This conclusion is based on the molecular size of specifically labeled polypeptides, cross-linking to PS-1 and PS-2 as shown by immunoprecipitation with PS-1 and PS-2 specific antibodies, and reduced binding to membranes derived from PS-1 gene-targeted embryos.

Compound C labels both N- and C-terminal PS-1 fragments with similar intensity. The photoaffinity ligand employed is monovalent, and the binding isotherms for THP-1 membranes are consistent with a single high affinity binding site. These results suggest that the compound is labeling an area of the PS-1 complex of close proximity of N- and C-terminal fragments. Interestingly, modeling studies suggest that two highly conserved Asp residues (Asp-257, Asp-385; PS-1 numbering) necessary for PS-1 and PS-2 function are in close proximity in adjacent transmembrane loops comprised of N- and C-terminal PS fragments (15, 16). The positive correlation between cellular potency and membrane binding suggests that the compounds can easily penetrate and possibly insert into the transmembrane domains of PS-1. If PS-1 itself is an aspartyl protease required for  $\gamma$ -secretase cleavage, it is tempting to speculate that these inhibitors bind in the transmembrane region near the putative catalytic site.

It should be noted that Wolfe *et al.* (15) suggested that the PS-1 holoprotein is a zymogen and requires proteolytic activation for biological activity. In this respect, we currently aim to address the question of whether cleavage of PS-1 and incorporation into the high molecular weight PS-1 complex are prerequisites for binding and cross-linking. In addition, the binding and cross-linking to PS-1 familial AD mutants are currently being analyzed. It should be noted that Compound A inhibits A $\beta$  production in human fibroblasts derived from the familial AD A246E mutation followed transient transfection with  $\beta$ APP.

PS-1 and PS-2 are the major molecular target for this series of  $\gamma$ -secretase inhibitors. The 30- and 20-kDa specifically labeled polypeptides are, based on immunological characterization, N- and C-terminal PS-1 fragments. Moreover, the 25-kDa specifically cross-linked polypeptide was identified as the C-terminal fragment of PS-2. Interestingly, little if any labeling of the N-terminal fragment of PS-2 was observed. This result suggests that the compound binding pockets in PS-1 and PS-2 are distinct. The involvement of PS-2 in binding and cross-linking is consistent with the observation of residual specific binding to membranes derived from PS-1<sup>-/-</sup> animals. The compounds employed in this study completely blocked A $\beta$  formation in mammalian cells,<sup>2</sup> whereas A $\beta$  production in cells derived from PS-1 gene-targeted mice is reduced only by 50–70%

(14), and no inhibition is observed in cells derived from single PS-2 knockout animals (24). Furthermore, A $\beta$  was reported as undetectable in cells derived from both PS-1 and PS-2 double knockout animals.<sup>3</sup> These results are consistent with binding of  $\gamma$ -secretase inhibitors to PS-1 and PS-2.

In summary, the results of the present study further link the involvement of PS-1 and PS-2 to  $\gamma$ -secretase action since small molecule  $\gamma$ -secretase inhibitors bind to PS-1 and PS-2. These studies define PS-1 and PS-2 as the molecular target for  $\gamma$ -secretase inhibitors developed for the pharmacological treatment of AD.

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